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Identification of superantigen genes *speM*, *ssa*, and *smeZ* in invasive strains of beta-hemolytic group C and G streptococci recovered from humans

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Abstract

Group C and G *Streptococcus dysgalactiae* subspecies *equisimilis* (GCSE and GGSE) cause a substantial percentage of invasive disease caused by beta-hemolytic streptococci. To determine whether *Streptococcus pyogenes* superantigen (SAg) genes commonly exist within these organisms, 20 recent invasive GCSE and GGSE human isolates and one group G *Streptococcus canis* human isolate were tested for the presence of SAg genes *speH*, *speJ*, *speL*, *speM*, *ssa* and *smeZ* by polymerase chain reaction (PCR). Prior to this work, sequence-based evidence of the *speM*, *ssa*, and *smeZ* genes in GCSE, GGSE, and *S. canis* had not been documented. Eleven of the 21 isolates were PCR-positive for the presence of one to two of the SAgs *speM*, *ssa*, or *smeZ*, with four of these isolates carrying *ssa*+*speM* or *ssa*+*smeZ*. No isolate was positive for *speH*, *speJ* and *speL*. All six *ssa*-positive GGSE strains harbored the *ssa3* allele, previously only found among *S. pyogenes* strains. All three *smeZ*-positive GGSE isolates carried one of two *smeZ* alleles previously only found within *S. pyogenes*, however the single *S. canis* isolate carried a new *smeZ* allele. All five GCSE and GGSE *speM*-positive isolates harbored a newly discovered *speM* allele. The identification of these SAgs within *S. dysgalactiae* subsp. *equisimilis* and *S. canis* with identical or near-identical sequences to their counterparts in *S. pyogenes* suggests frequent interspecies gene exchange between the three beta-hemolytic streptococcal species.

Keywords: Group C streptococcus; Group G streptococcus; Superantigen; Streptococcal pyrogenic exotoxin

1. Introduction

Group C and G streptococci (GCS and GGS) are commonly regarded as commensals because they are often found in association with the normal flora of human skin, pharynx and the intestine [1,2]. In contrast, the closely related group A streptococcus (*Streptococcus pyogenes* or GAS) is a human pathogen that commonly causes a wide range of non-invasive and invasive infections. However, there has been an increasing number of reports showing the isolation of GCS and GGS from human infections with clinical manifestations similar to infections caused by GAS and in some instances GCS and GGS have been implicated in severe invasive infections such as necrotizing fasciitis and toxic shock syndrome [2,3–9]. Recent preliminary data from Active Bacterial Core Surveillance (ABCs), part of the Emerging Infections Program Network in the state of Georgia, suggests that group G and C streptococci or *Streptococcus dysgalactiae* subspecies *equisimilis* (GGSE and GCSE respectively) constitute more than 60% of invasive GGS and GGC human infections, and account for approximately 5% of the invasive burden due to beta-hemolytic streptococci in this ABCs region (unpublished data).

GCSE and GGSE express several virulence determinants initially identified in GAS. They express a hyaluron-

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ic acid capsule, anti-phagocytic M protein, streptolysins O and S, streptokinase, C5a peptidases, and binding proteins for fibronectin, plasminogen, and immunoglobulin [10]. Besides these factors, GAS strains express a large number of distinct, but structurally related streptococcal pyrogenic exotoxins (SPEs) [10–12]. These effectors belong to the family of superantigens (SAgs) [13–15]. These SAgs are capable of binding to the major histocompatibility complex II molecules on antigen presenting cells and T cell receptors [14], leading to the release of large amounts of cytokines. These cytokines have been shown to play major roles in fever, tissue destruction, shock, hypotension, and organ failure associated with invasive streptococcal infections [12,15–18].

Most reported SAg genes are located on integrated phage genomes, a situation that is putatively conducive to genetic transfer among different GAS *emm* types as well as other streptococcal species [19–22]. Presently, several SAg genes of GAS have been described [11,12,19,23–27]. GAS pyrogenic exotoxin A, C and G determinants have been found in human pathogenic GGSE [20,28], and SAg genes with high homology to *speH*, *speI*, and *speL* of GAS have been identified in group C *Streptococcus equi* [22,29]. Since SAg genes appear to be randomly distributed among streptococcal isolates and other related species, it appeared likely that other SAg genes are present within GGSE and GCSE. Therefore we screened a set of 20 diverse *S. dysgalactiae* subsp. *equisimilis* invasive isolates and a single *Streptococcus canis* human invasive isolate (the only one available to us from Georgia surveillance at this time) for the presence of six GAS SAgs that have not been previously documented among these two species.

2. Materials and methods

2.1. Bacterial isolates

A set of 21 consecutive M protein gene (*emm*) typeable group C and G beta-hemolytic streptococcal isolates (GCS and GGS) were obtained from invasive streptococcal infections in metropolitan Atlanta identified by the Georgia ABCs system from mid 2002 through early 2003. One hundred-twenty GAS isolates (invasive and non-invasive) recovered during the past five years from a variety of human sources and geographic sites (within and outside of the USA) were used to verify that the SAgs could be readily screened among GAS. All isolates used in this study were identified to the species level by previously described phenotypic methods [30].

2.2. emm typing

emm typing and *emm* subtype determination were performed for all isolates as previously described [31,32].

Table 1
Primers used for the amplification of SAg genes from DNA of group A, C and G streptococcal isolates

Gene	Primer sequence
speH	5′-CACATATTGATAAGAAAATCTACAGC forward
	5′-GAAATTGAGTTGAGTCTATTCTCTCG reverse
speJ	5′-GTTATAATAATCTTTCATGGGTACGG forward
	5′-CTTTCATGTTTATTGCCATTGATCGC reverse
speL	5′-TTAGGATGGTTTCTGCGGAAGAGAC forward
	5′-TTCCTCTTTCTCGCCTGAGCCGTG reverse
speM	5′-GCTCTATACACTACTGAGAGTGTC forward
	5′-CATATCAATCGTTTCATTATCTG reverse
ssa	5′-GTGCACAATTATTATCGATTAGTG forward
	5′-GGTGAACCTCTATAGCTATAGCTGAAG reverse
smeZ	5′-GAAGTAGATAATAATTCCCTTCTAAGG forward
	5′-AGTCAATTTCTATATCTAAATGCCC reverse

Primer sequence pairs were designed from streptococcal genome sequencing projects (accession numbers AE004092, NC002737 and NC003485).

2.3. Polymerase chain reaction (PCR) amplification and sequencing of SAg genes

Crude chromosomal DNA extracts were prepared using mutanolysin and hyaluronidase as previously described [31]. Extracts were screened by PCR for SAg genes using primers indicated in Table 1. PCR products were sequenced with an ABI 3100 Genetic Analyzer using the same primers that were used for amplification.

2.4. Flanking sequence of the smeZ gene in S. canis isolate

The 5′ and 3′ ends of the *smeZ* structural gene and additional sequence flanking *smeZ* within the single *S. canis* isolate were obtained by single primer PCR as described previously [33]. Briefly, a denaturation at 94°C for 4 min was followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 3 min. This was followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 2 min; then, a final extension step at 72°C for 7 min. Primer 5′-GAC AAT AAA TCT TTC AAT ATA GAT-3′ (anneals to bases 643–666 of *smeZ*) was used for a single PCR amplification of the *smeZ* 3′ region and downstream region which was sequenced with primer 5′-GGGCATTTAGATATAGAAATTGACT-3′ (anneals to bases 673–679 of *smeZ*). The 5′ end of smeZ and upstream region was amplified with primer 5′-AGC ATA TCT AAC ATC AAG TTT C-3′ (anneals to *smeZ* complement, bases 395–416) and sequenced with primer 5′-AGT CAC TAA GTT ATG ACT GGT-3′ (bases 372–392 of *smeZ*). The sequence data obtained from single primer PCR-generated template was reconfirmed using sequence obtained from conventional PCR products generated with two primers from strain 4074-03 chromosomal DNA.

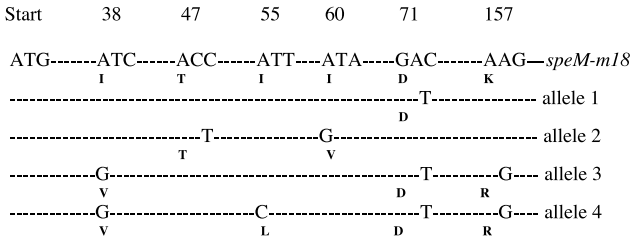

Fig. 1. Four alleles of *speM* gene found during this work. Numbers along the upper portion of the figure indicate the codons; amino acids are indicated with bold letters. All nucleotides different from those in the *speM* allele (*speM18*) of GAS serotype M18 strain MGAS8232 [27] are shown. Alleles *speM18*, *speM1* (accession number AJ560765), *speM2* (accession number AJ56060766) and *speM3* (accession number AJ560767) are from GAS isolates, while *speM4* (accession number AJ557010) is from GCSE and GGSE.

2.5. Sequence analysis

New nucleotide sequences not found in the GenBank were deposited in NCBI database under the accession numbers: AY344227 (*S. canis smeZ25* allele and flanking region), AJ557010 (*S. dysgalactiae* subsp. *equisimilis*; *speM4* allele), AJ560765 (*speM1* allele), AJ560766 (*speM2* allele), AJ560767 (*speM3* allele) and AJ560764 (*speL1* allele).

3. Results and discussion

GCSE and GGSE are not commonly regarded as invasive human pathogens. However, the increasing frequency of recovery of these organisms from human invasive disease cases, and the discovery of SAgs *speA*, *speC*, and *speG* in GGSE [20,28] led us to screen for additional SAgs within GGSE and GCSE. Since most known SAg genes appear to be located on mobile elements that can be transferred between different streptococcal species, it suggests that additional SAgs found in GAS may be found in GCS or GGS.

3.1. speH, speJ, *and* speL

All 21 GGS and GCS were PCR-negative for the presence of these *speH*, *speJ*, and *speL* (Table 1) using primers that anneal to structural gene sequences. This indicates that either these genes do not exist within these isolates, or that these isolates lack *spe* gene sequences highly similar to one or both of the two primers in the primer sets used.

3.2. speM

We first wanted to determine whether *speM* was likely to be widely disseminated among GAS strains. Examination of 120 GAS isolates comprising 28 *emm* types revealed that 11 isolates representing six types (*emm* types 4 [one of 10 tested], 6 [one of six tested], 18 [two of two tested], 22 [one of eight tested], 28 [one of five tested], 41 [three of three tested]) were positive for both *speL* and *speM*. The *speM* sequence found within our two type *emm18* isolates was identical to the single allele previously described [27]. In addition, we identified three new *speM* alleles from GAS strains (Fig. 1, alleles 1–3). In all 11 speM-positive GAS isolates *speL* and *speM* were closely linked, since flanking primers (to the type M18 open reading frames (ORFs) spyM18_1237 and spyM18_1240) amplified fragments of the appropriate size (Fig. 2) which were shown by nested PCR to contain the *speL* and *speM* genes. Smoot et al. [27,34] found *speM* within all

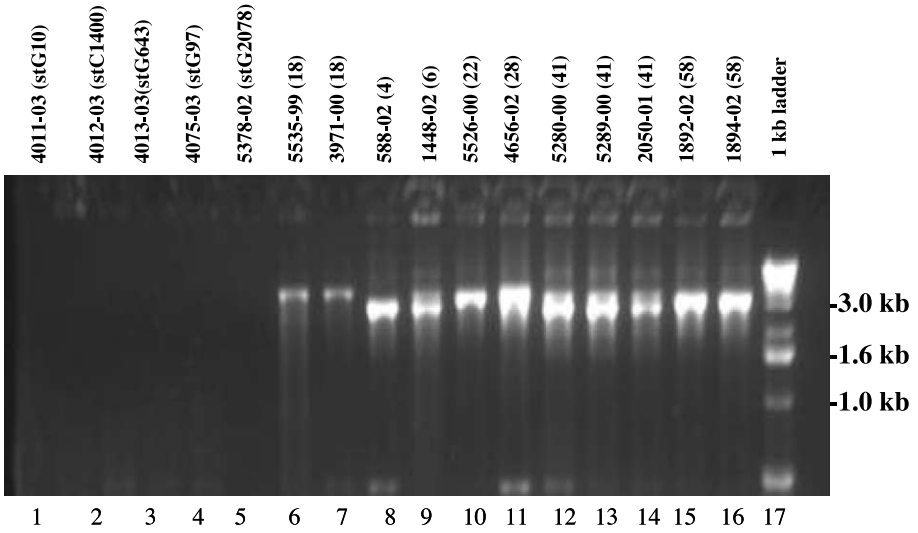

Fig. 2. PCR analysis indicating that *speL* flanks *speM* in diverse GAS strains but not GCS and GGS study isolates. Primers annealing to the ORFs flanking *speL* and *speM* genes in *S. pyogenes* serotype M18 were used for the amplification. Lanes 1–5, GCS and GGS isolates; lanes 6–16, GAS isolates from different *emm* types. The *emm* types of GAS isolates are indicated in parentheses. All GCS and GGS study isolates were additionally negative for *speL*-specific PCR.

Table 2
S. pyogenes SAg genes among invasive strains of group C and G *Streptococcus* recovered from humans

Isolate no.	*emm* type	Source	*speH, J, L*	*smeZ*	*speM*	*ssa*
3906-03	stG2078	blood	–	–	–	–
4011-03	stG10	blood	–	–	*speM18*	*ssa3*
4012-03	stC1400	blood	–	–	*speM18*	–
4013-03	stG643	blood	–	–	*speM18*	*ssa3*
4074-03	stG1389	joint	–	*smeZ25*	–	–
4127-03	stG643	blood	–	–	–	*ssa3*
4128-03	st245	blood	–	–	–	*ssa3*
3434-03	st245	blood	–	*smeZ17*	–	*ssa3*
3092-03	st245	blood	–	–	–	–
3853-03	stG480	blood	–	–	–	–
3963-03	stG480	blood	–	*smeZ2*	–	*ssa3*
4075-03	stG97.0	blood	–	–	*speM18*	–
5420-02	stG5420	blood	–	–	–	–
6328-02	stG643	blood	–	–	–	–
6330-02	stG652	blood	–	–	–	–
6320-02	stG6	blood	–	–	–	–
6317-02	stG653	blood	–	–	–	–
6282-02	stG2078	blood	–	*smeZ2*	–	–
6198-02	stG485	blood	–	–	–	–
5376-02	st245	blood	–	–	–	–
5378-02	stG2078	bone	–	–	*speM18*	–

All isolates are *S. dysgalactiae*, subsp. *equisimilis* except isolate 4074-03 which is a *S. canis*. Isolates 4012-03 and 6317-02 are GCS, while the other isolates are GGS.

33 type *emm18* isolates tested, but did not find *speM* within individual isolates of *emm* types 1–6, 11, 12, 22, 28, 49, 77, and 89. It is not surprising that these authors did not find *speM* within types *emm4*, *emm22*, and *emm28* since we found that the majority of isolates within these types were negative for *speM*. In agreement with these authors, we did not detect *speM* or *speL* within *emm* types 11 (three isolates), 12 (12 isolates), 77 (one isolate), and 89 (four isolates).

It has previously been shown that *speM* and *speL* are located on a prophage-like element in type *emm18* GAS [27]. Five of 21 (24%) GCS and GGS isolates (one GCSE and four GGSE) were *speM* PCR-positive, yielding a 610 bp product (corresponding to bases 50–660 of the GAS 710 base structural gene), however attempts to amplify *speL* in all 21 GGS and GCS isolates were unsuccessful using either *speL*-specific primers or phage-specific primers flanking the GAS *speL* and *speM* genes. The five *speM*-positive isolates identified in this study yielded amplicons with an identical partial *speM* sequence (consisting of eight signal sequence codons and 196 codons encoding mature protein). This *speM* sequence (allele 4 in Fig. 1) was highly similar to the full-length *speM* (consisting of 24 signal sequence codons and 216 mature protein codons) from strain MGAS8232 [34] (four base changes: three conservative substitutions (14 Val to Leu, 39 L to I, 141 (R to K) and one synonymous substitution). *speM4* from these five *S. dysgalactiae* subsp. *equisimilis* isolates was also found to be 97.4% identical to a previously reported *speM* allele from *S. dysgalactiae* subsp. *dysgalactiae* (designated *sdm* by these authors) [35].

3.3. ssa

Previous studies demonstrated that *ssa* is widely spread among *S. pyogenes* and was found within about 12.5% of 80 distinct clonal lineages [36]. This group [36] additionally found that *ssa* was absent from 79 GGS and GCS, although the species of these GGS and GCS were not communicated in this report. We found that six of 21 GGS and GCS (all *S. dysgalactiae* subsp. *equisimilis*) were PCR-positive for the presence of *ssa* and harbored a sequence identical to bases 46–769 of the 780 base *ssa3* allele that is widely distributed among diverse GAS strains [36].

3.4. smeZ

Of the four *smeZ*-positive GGS and GCS isolates, two were found to carry *smeZ2*, one isolate carried *smeZ17*, and the single *S. canis* isolate (4074-03) carried the new *smeZ25* allele (Table 2). This allele differs from its closest match, *smeZ17*, by three unique nucleotide substitutions relative to sequence encoding the processed protein (codon 36 synonymous, codon 143 Met to Thr, codon 152 Ser to Asn). The predicted processed forms of SmeZ17 and SmeZ25 demonstrate the rather wide degree of allelic variation inherent to this determinant [37] since they share only about 94% amino acid sequence identity with SmeZ2 and have 10–11 substitutions relative to SmeZ2 distributed between residues 23 and 177 of the 210 amino acid protein.

Since *smeZ* is known to be a chromosomal determinant

in GAS [11,34,37], additional sequence flanking the *smeZ25* structural gene was obtained from the single *S. canis* isolate employing single primer PCR. No ORFs greater than 30 codons were found within the 415 bases downstream of *smeZ25*. The sequence of bases 1–252 immediately downstream of the *smeZ* stop codon was 94.4% identical to corresponding non-annotated sequences downstream of *smeZ* in the types M1, M3, and M18 genomes (see accession numbers AE006622, AE014169, and AE010110 respectively). Bases 253–415 did not show high levels of significant identity to any known sequences. The sequence corresponding to the signal sequence and putative promoter region was highly similar to corresponding sequences from the M1, M3, and M18 genome sequences. The 197 base sequence immediately upstream of *smeZ25* was nearly identical to corresponding sequences from the M1, M3, and M18 genomes. Bases 198–306 showed no significant homologies to streptococcal sequences. These flanking sequences are consistent with integration of a 1080 bp fragment of *S. pyogenes* chromosomal DNA between coordinate base positions −252 and +198 relative to the *smeZ* structural gene in this *S. canis* isolate.

3.5. Conclusions

Evidence has suggested that *S. pyogenes* SAgs play key roles in the development of severe invasive diseases such as toxic shock and necrotizing fasciitis [10]. Additionally, the correlation between the recoveries of *speL*- and *speM*-positive GAS isolates from acute rheumatic fever (ARF) patients, and the presence of *speL* and *speM* antibodies in these patients suggests that these SAgs play role(s) in this disease [27]. Increasing reports of invasive GCS and GGS recovered from humans and reports of the presence of GAS SAg genes in these organisms indicates the possibility that more virulent forms of these organisms have emerged through the acquisition of SAg genes from *S. pyogenes*. Eleven of the 21 invasive GGS and GCS screened during this study were positive for the presence of one or more of these genes, with four of them positive for the presence of two SAg genes formerly thought to be exclusive to *S. pyogenes*. These data suggest that the transfer of these genes from *S. pyogenes* to *S. dysgalactiae* subsp. *equisimilis* has occurred frequently.

Previous to this work, sequence-based evidence demonstrating the presence of *ssa*, *smeZ* and *speM* genes in GGSE and GCSE had not been provided. The *ssa* gene was previously detected by DNA hybridization among human isolates of GCSE and GGSE [28] but attempts to further confirm these results using PCR were unsuccessful. Here we have reported the initial identification of the *ssa*, *smeZ* and *speM* genes of GGSE and GCSE isolated from humans. Additionally, we have demonstrated a new *smeZ* allele in a human isolate of *S. canis*, demonstrating the wide dissemination of the GAS *smeZ* gene within yet another streptococcal species.

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